Kidney International, Vol. 60 (2001), pp. 1354–1365

LF15-0195 prevents the induction and inhibits the progression of rat anti-GBM disease

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LF15-0195 prevents the induction and inhibits the progression of rat anti-GBM disease.

Background. LF15-0195 is a novel immunosuppressant that is currently in phase II clinical trials for the treatment of vasculitis. This study examined whether LF15-0195 could suppress the induction and progression of rat anti-glomerular basement membrane (anti-GBM) glomerulonephritis.

Methods. Rapidly progressive glomerulonephritis was induced in primed rats by the administration of anti-GBM serum. In the first experiment, LF15-0195 was given daily by subcutaneous injection (days 0 to 14) to treat the induction of anti-GBM disease analyzed at day 14. In a second experiment, rats received LF15-0195 as an intervention treatment from days 7 to 28 (continuous therapy) or days 7 to 12 (pulse therapy) to treat the progression of disease assessed at day 28.

Results. Continuous LF15-0195 treatment during the induction of anti-GBM disease (experiment 1) prevented proteinuria and loss of renal function, and markedly reduced histological kidney lesions and renal fibrosis. LF15-0195 also reduced kidney leukocyte infiltrate, urine excretion of interleukin-1β (IL-1β) and transforming growth factor-β (TGF-β), and the serum antibody response, but not kidney deposition of Ig and C3. When LF15-0195 treatment was initiated at day 7, both continuous and pulse therapy partially inhibited disease progression by suppressing the loss of renal function, interstitial macrophage and T-cell accumulation, tubular cell proliferation, and renal fibrosis.

Conclusion. LF15-0195 prevents the induction and suppresses the progression of rat anti-GBM disease through multiple mechanisms of action, suggesting that this drug may have significant therapeutic potential in human glomerulonephritis. The similar efficacy of continuous and pulse intervention treatment in this model indicates that short-term LF15-0195 treatment may achieve optimal benefit without prolonged bone marrow suppression.

Key words: immunosuppression, vasculitis, progressive glomerular disease, proteinuria, renal fibrosis, tubular cell proliferation.

Received for publication November 14, 2000
and in revised form May 9, 2001
Accepted for publication May 16, 2001

LF15-0195 is a new immunosuppressant that is structurally related to the spergualin family of drugs that includes tresperimus and 15-deoxyspergualin (DSG) [1]. This family has been shown to be more effective than the popular immunosuppressants cyclosporine A (CsA), FK506, and rapamycin at prolonging the survival of tissue grafts [2–4] and eliciting fewer adverse side effects [5–7]. The recently developed LF15-0195 has increased metabolic resistance and improved activity compared with other family members, resulting in greater potency [1]. In a rat model of heart allotransplantation, a 10-day postsurgical treatment with LF15-0195 (2.5 mg/kg) achieved a mean survival of 80 ± 30 days, which was significantly better than DSG (6 mg/kg), with a mean survival of 38 ± 30 days [1]. LF15-0195 is now being investigated as a potential therapeutic drug for treating both acute and chronic inflammatory diseases.

The immunosuppressive properties of the spergualin family are known to be different than the ones of the CsA family [8]; however, the mode of action of these spergualin-related drugs is still poorly defined. In vitro and in vivo studies have shown that DSG can suppress T-cell and macrophage proliferation, cytotoxic T-lymphocyte (CTL) generation, B-cell maturation, antibody formation, antigen presentation, adhesion molecule expression, and cytokine production [9]. It has been suggested that some of these effects may be due to an inhibition of nuclear factor-κB activity (NF-κB) through drug interaction with members of the heat shock protein 70 (HSP-70) family [10, 11]. Recent evidence also indicates that tresperimus is capable of inducing specific CD4+ suppressor cell activity, thereby inhibiting T-cell–mediated injury [12].

LF15-0195 may have therapeutic potential for treating human glomerulonephritis. In a recent open clinical investigation, DSG was shown to be effective at suppressing proteinuria in several patients with human proliferative glomerulonephritis, including two with IgA nephropathy [13]. Animal studies have shown that DSG

can inhibit the development of both the humoral and the aggressive cellular immune responses associated with the evolution of rapidly progressive immune-mediated kidney disease [9] and can inhibit the activity of intrinsic renal cells [14]. DSG treatment during the induction of rat anti-GBM disease reduces proteinuria and hematuria, prevents a loss in renal function, and improves the renal histology [15]. However, the potential for DSG as an intervention treatment in this model has not been assessed.

The goal of this study was to evaluate whether subcutaneous LF15-0195 treatment is effective in suppressing the induction of rat anti-GBM disease and the progression of established disease. Our assessment included an analysis of both humoral and cellular immune responses and a comparison between continuous LF15-0195 treatment and short-term "pulse" therapy.

METHODS

Materials

LF15-0195 was provided by Fournier Laboratoires (Daix, France). Enzyme-linked immunosorbent assay (ELISA) kits detecting rat interleukin-1β (IL-1β; Endogen, Woburn, MA, USA) and rat transforming growth factor-β1 (TGF-β1; Promega, Madison, WI, USA) were used for urine cytokine analysis. Monoclonal antibodies (mAbs) used in this study were OX-1, anti-rat CD45R (leukocyte common antigen); ED1, anti-rat CD68 (macrophage-specific antigen); R73, anti-rat T-cell receptor (nonpolymorphic determinant); NDS-61 and OX-39, anti-rat CD25 (IL-2 receptor α-chain); 1A4, anti-α-smooth muscle actin (Sigma, St. Louis, MO, USA); HWD1.1, anti-collagen III (BioGenex, San Ramon, CA, USA); Bu20a, anti-bromodeoxyuridine (Dako, Carpinteria, CA, USA); and 73.5, anti-human CD45, which does not react with rat tissues. Polyclonal primary antibodies used in this study were fluorescein isothiocyanate (FITC)-conjugated rabbit anti-sheep Ig, FITC-conjugated rabbit anti-rat IgG, and FITC-conjugated rabbit anti-rat C3 (all from Sigma). Antibodies not purchased from commercial sources were produced by cell culture of hybridomas obtained from the European collection of cell cultures (ECACC).

Animal model and treatment

Male inbred Sprague-Dawley rats were obtained from Monash Animal Services (Melbourne, Australia). Anti-GBM disease was induced in Sprague-Dawley rats as previously described [16]. Briefly, rats were immunized with 5 mg of normal sheep IgG in Freund's complete adjuvant (FCA) and injected intravenously with sheep anti-rat GBM serum seven days later. Disease was induced in groups of eight rats that received daily subcutaneous injections of either normal saline or LF15-0195 according to the experimental protocol. Experiments were designed to determine whether LF15-0195 treatment could prevent the induction of anti-GBM disease (experiment 1) and the progression of established anti-GBM disease through continuous treatment (experiment 2a) and pulse therapy (experiment 2b). LF15-0195 was administered at a dose that provided maximum immunosuppression without detrimental side effects for the duration of the experiment. In experiment 1, groups received either saline or LF15-0195 (4 mg/kg/day) from day 0 until being killed at day 14. In experiment 2a, groups received either saline or LF15-0195 (1 mg/kg/day) from day 7 until being killed at day 28. An additional group was killed at day 7 in this experiment to determine the extent of kidney pathology prior to treatment. In experiment 2b, groups were administered with either saline or LF15-0195 (4 mg/kg/day) from days 7 to 12 and were killed at day 28. Two hours prior to being killed, all rats received an intraperitoneal injection of saline containing bromodeoxyuridine (BrdU; 50 mg/kg), which incorporated into proliferating cells.

In addition, a skin delayed-type hypersensitivity (DTH) response was assessed in groups of eight rats. Animals were primed with 5 mg sheep IgG in FCA and challenged 14 days later with intradermal injections of 100 μg of sheep IgG and bovine serum albumin (BSA). Two hours prior to antigen challenge, rats received a single subcutaneous injection of either saline or LF15-0195 (4 mg/kg). Skin swelling at the antigen injection site was measured by micrometer calipers after 24 hours.

Hematology assessment

Whole blood cell counts were performed on a Cell-Dyn 3500 automated cell counter (Abbott Laboratories, Abbot Park, IL, USA) using heparinized blood collected from tail veins.

Biochemical analysis of blood and urine

Urine was collected over 24 hours from rats housed in metabolic cages. Serum was collected from whole blood obtained from the tail vein of anesthetized rats. Urine protein, urine creatinine, and serum creatinine were analyzed by the Department of Biochemistry at the Monash Medical Centre. Commercial ELISA kits were used to measure IL-1β (Endogen) and total TGF-β1 (Promega) in rat urine samples. The levels of anti-sheep IgG in rat serum were analyzed by ELISA as previously described [15].

Immunohistochemistry

Kidney deposition of sheep Ig, rat IgG, and complement C3 were detected on unfixed cryostat tissue sections (4 μm) by immunofluorescence staining with FITC-conjugated rabbit polyclonal antibodies. Semiquantitation of immunofluorescence staining was performed by a blinded antibody titration method [15]. Immunostaining

for kidney leukocytes, proliferating cells, myofibroblasts and collagen was performed with monoclonal antibodies on either paraformaldehyde-lysine-periodate (PLP) fixed cryostat sections (6 μm) or formalin-fixed paraffin sections (4 μm) using a three antibody layer immuno-peroxidase or immuno-alkaline phosphatase detection method, as previously described [17]. Sections immunostained for leukocytes and proliferating cells were counterstained with periodic acid-Schiff (PAS) reagent. The number of glomerular leukocytes was counted under high power (×400) in 20 glomerular cross-sections per animal. The number of interstitial leukocytes in the cortex was determined by counting the number of stained cells in 30 consecutive high-power fields (×250) by means of a 0.02 mm^2 graticule fitted in the eyepiece of the microscope. These fields progressed from the outer to inner cortex, avoiding only large vessels, glomeruli, and immediate periglomerular areas. No adjustment of the cell count was made for tubules or the luminal space. Proliferating tubular cells labeled with BrdU were assessed by counting the number of stained nuclei in 500 randomly selected cortical tubules per section. Kidney cortex expression of collagen III and α-smooth muscle actin was recorded by digital camera and evaluated as a percentage of area immunostained using image analysis software (Image J; Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA). Glomerular staining and constitutive expression of α-smooth muscle actin and collagen by kidney vessels was excluded from the image assessment.

Pathology assessment

Kidney pathology was assessed on 3 μm paraffin sections of formalin-fixed tissues stained with PAS and hematoxylin. Histopathology scoring was performed on blinded slides by a pathologist. Segmental glomerular lesions (proliferation/necrosis/sclerosis) were assessed in 50 glomeruli/kidney by scoring the glomerular area affected: 0 = none; 1 = 1 to 25%; 2 = 25 to 50%; 3 = 50 to 75%; and 4 = 75 to 100%. Glomerular crescents were defined as two or more cell layers within Bowman's space and were evaluated as the percentage of glomeruli with crescents per kidney. Tubulointerstitial lesions were determined by the expansion of the interstitial area and damage to tubules (tubulitis, dilation, atrophy, casts) and were graded as follows: 0 = no lesions; 1 = occasional mild lesions; 2 = intermittent moderate lesions; and 3 = frequent severe lesions.

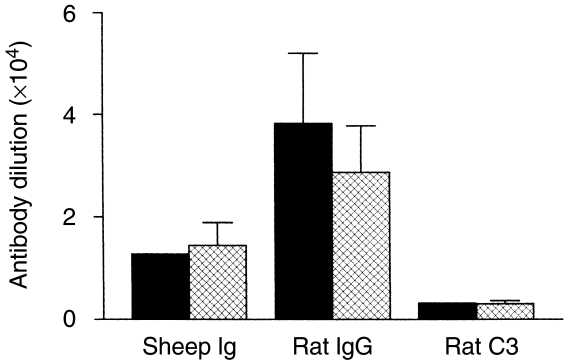


Fig. 1. LF15-0195 does not affect glomerular deposition of antibody or complement C3 during anti-glomerular basement membrane (GBM) disease. Glomerular deposition of sheep Ig, rat IgG, and rat C3 were detected by immunofluoresence staining of kidney sections at day 14 of disease. Serial antibody dilutions (twofold) were used to determine the end point of immunofluorescence. LF15-0195 treatment did not affect the deposition of Ig or C3. Data are mean ± SD, $N = 8$. Symbols are: (■) saline; (▩) LF15-0195.

Statistical analysis

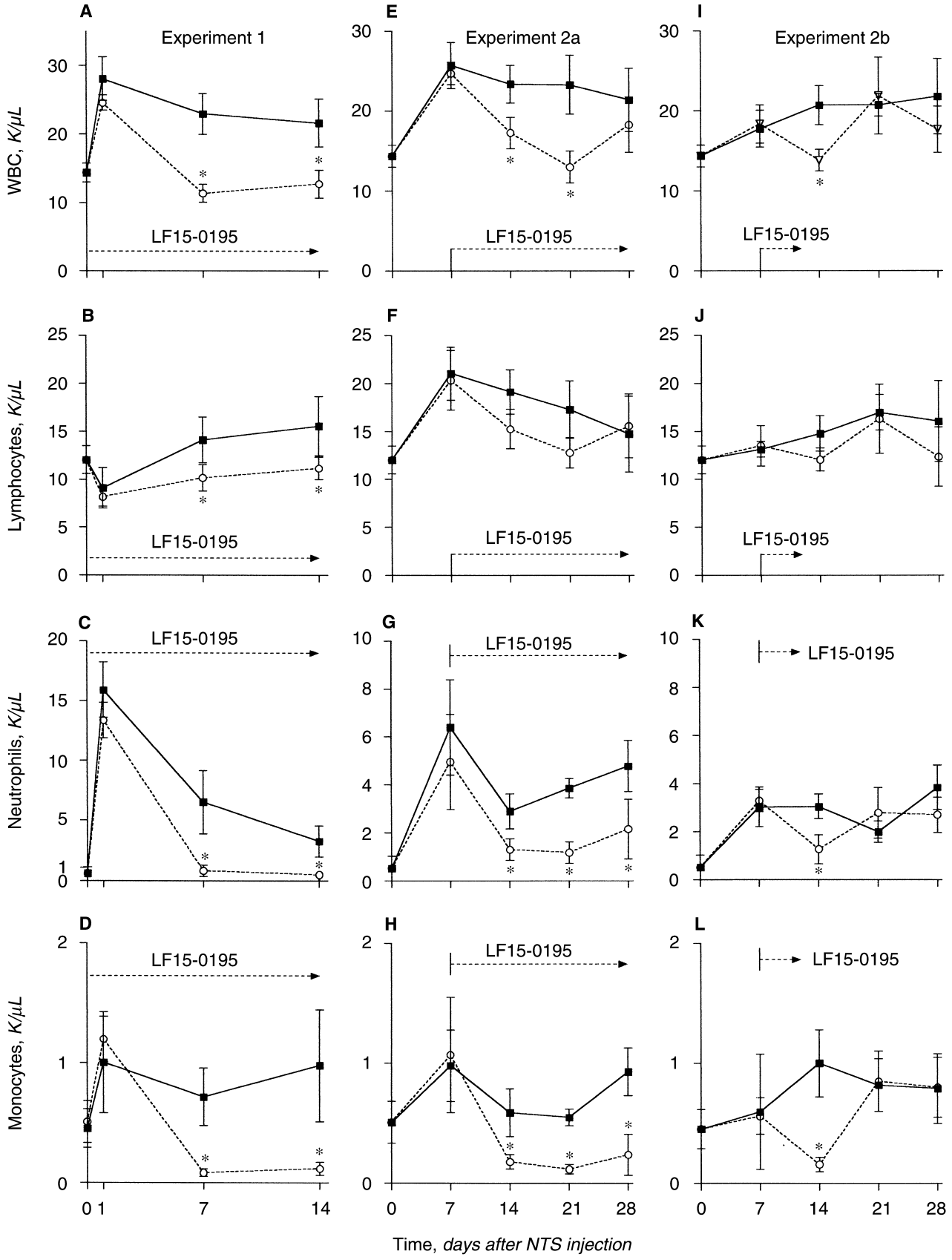
Statistical differences between two comparative groups were analyzed by the unpaired Student *t* test (parametric data) or the Mann-Whitney test (nonparametric data). Data was recorded as the mean ± standard deviation (SD) and values of $P < 0.05$ were considered significant. All of the analyses were performed using the statistical software in GraphPad Prism 3.0 (GraphPad Software, San Diego, CA, USA).

RESULTS

LF15-0195 treatment prevents the induction of anti-GBM disease

In this rat model of anti-GBM disease, induction of renal injury involves the glomerular deposition of sheep anti-GBM antibody followed by deposition of complement and rat IgG. Immunofluorescence staining at day 14 of anti-GBM disease showed similar glomerular levels of sheep Ig, rat IgG, and complement C3 deposited in saline-treated and LF15-0195–treated rats (Fig. 1). In contrast, serum obtained from LF15-0195–treated rats at day 14 of disease had reduced levels of anti-sheep Ig

Fig. 2. LF15-0195 suppresses white blood cell (WBC) numbers during anti-GBM disease. Symbols are: (■) saline; (○) LF15-0195. WBCs (lymphocytes, neutrophils, monocytes) were increased in saline-treated rats following the induction of anti-GBM disease. Daily LF15-0195 treatment, commencing at the induction of disease (experiment 1), reduces the rise in WBC counts at days 7 and 14 compared with saline (*A–D*). Continuous intervention treatment with LF15-0195, beginning at day 7 of disease (experiment 2a), reduces the neutrophils and monocytes compared to saline treatment (*E–H*). Pulse treatment with LF15-0195 from days 7 to 12 (experiment 2b) temporarily reduces the neutrophils and monocytes at day 14 of disease compared with saline (*J–L*). Data are mean ± SD, $N = 8$; $^*P < 0.005$ vs. saline-treated.


A
Experiment 1
E
Experiment 2a
I
Experiment 2b
WBC, K/μL
B
F
J
Lymphocytes, K/μL
C
G
K
Neutrophils, K/μL
D
H
L
Monocytes, K/μL
LF15-0195
Time, days after NTS injection

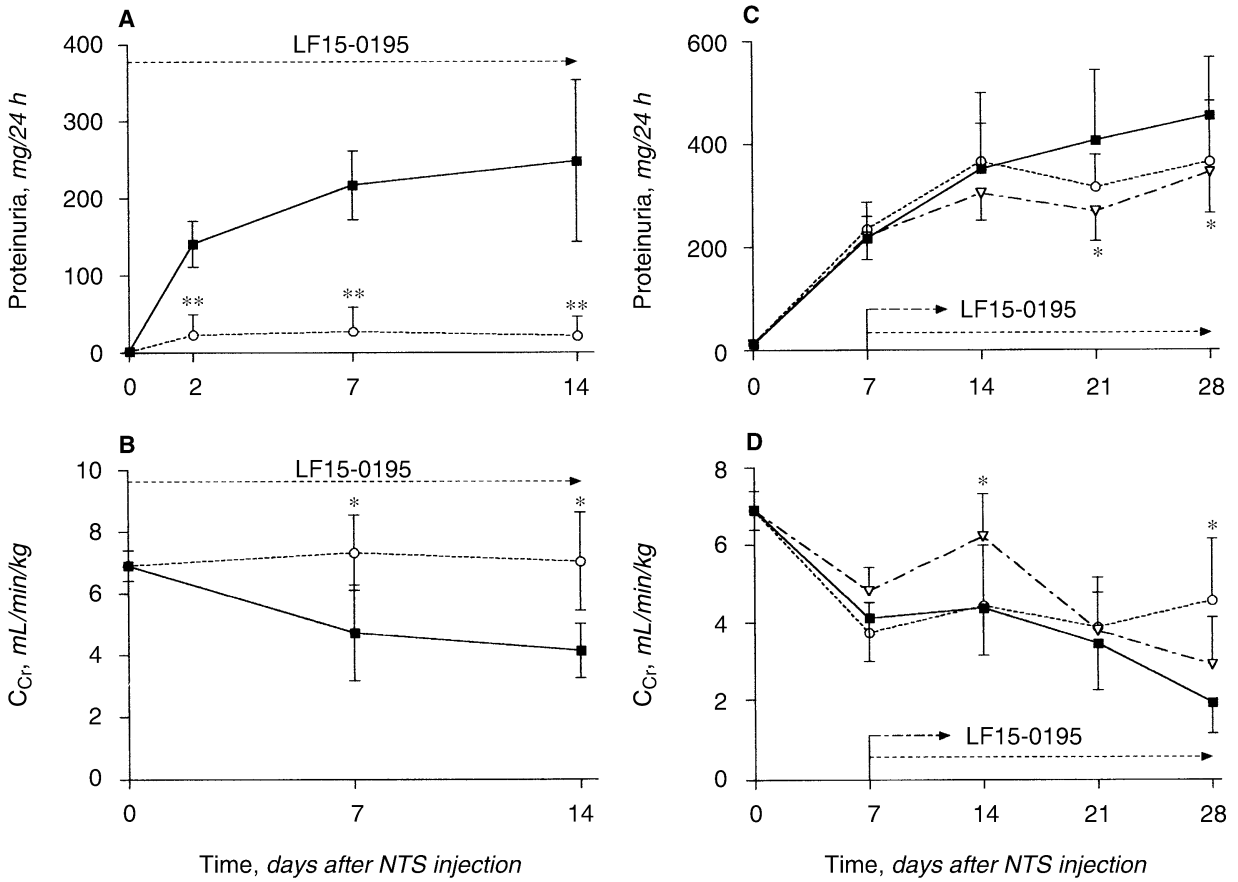


Fig. 3. LF15-0195 inhibits proteinuria and loss of renal function in anti-GBM disease. Daily LF15-0195 treatment from the initiation of anti-GBM disease prevents the development of proteinuria (*A*) and the decline in creatinine clearance (C_{Cr}) (*B*) associated with disease progression. LF15-0195 treatment beginning at day 7 of anti-GBM disease inhibits the progressive increase in proteinuria (*C*) and provides some resistance against the progressive reduction in creatinine clearance (*D*). Data are mean ± SD, $N = 8$; *$P < 0.05$; **$P < 0.005$ vs. saline treated. Symbols are: (■) saline treated; (○) LF15-0195 (in A and B) or continuous LF15-0195 (in C and D); (▽) pulse LF-15-0195.

(↓60%, $P < 0.05$) compared with saline-treated rats (data not shown).

During the induction of anti-GBM disease, circulating white blood cells (WBCs) were increased by 40 to 80% in rats given saline treatment (Fig. 2A). In comparison, LF15-0195–treated rats had reduced numbers of WBCs at days 7 and 14 that were similar to normal (Fig. 2A). Further analysis indicated that LF15-0195 reduced the increased numbers of blood lymphocytes, neutrophils, and monocytes at days 7 and 14 compared with saline treatment; however, only monocytes were reduced by LF15-0195 compared with normal animals (Fig. 2 B–D).

Induction of anti-GBM disease resulted in the rapid development of proteinuria and a reduction in creatinine clearance in saline-treated rats, which was prevented by LF15-0195 treatment (Fig. 3 A, B). At day 14, saline-treated rats had moderate histological kidney damage, including segmental glomerular proliferative lesions, glomerular sclerosis with occasional glomerular crescents, dilated and atrophic tubules with protein casts, and significant numbers of glomerular and interstitial infiltrating cells (Figs. 4A and 5 A–C). In comparison, rats that received LF15-0195 during disease induction showed almost no evidence of kidney pathology (Figs. 4B and 5 A–C). These rats had histologically normal glomeruli and tubules and few infiltrating cells in the interstitium.

The accumulation of kidney-infiltrating leukocytes, myofibroblasts, and extracellular matrix was associated with the development of anti-GBM disease in saline-treated rats. LF15-0195 largely suppressed the glomerular and interstitial leukocyte infiltrate seen in saline-treated kidneys at day 14 (Figs. 4D and 6). Similarly, LF15-0195 prevented the appearance of α-smooth muscle actin–labeled myofibroblasts in the interstitial and periglomerular regions of the kidney (Fig. 4 E, F) and an increased interstitial expression of collagen type III at day 14 compared with normal animals (microscopy observations).

Significant levels of IL-1β (2.01 ± 0.96 pg/24 h) and TGF-β (3.11 ± 1.52 pg/24 h) appeared in the urine of saline-treated rats at day 14 of anti-GBM disease, but

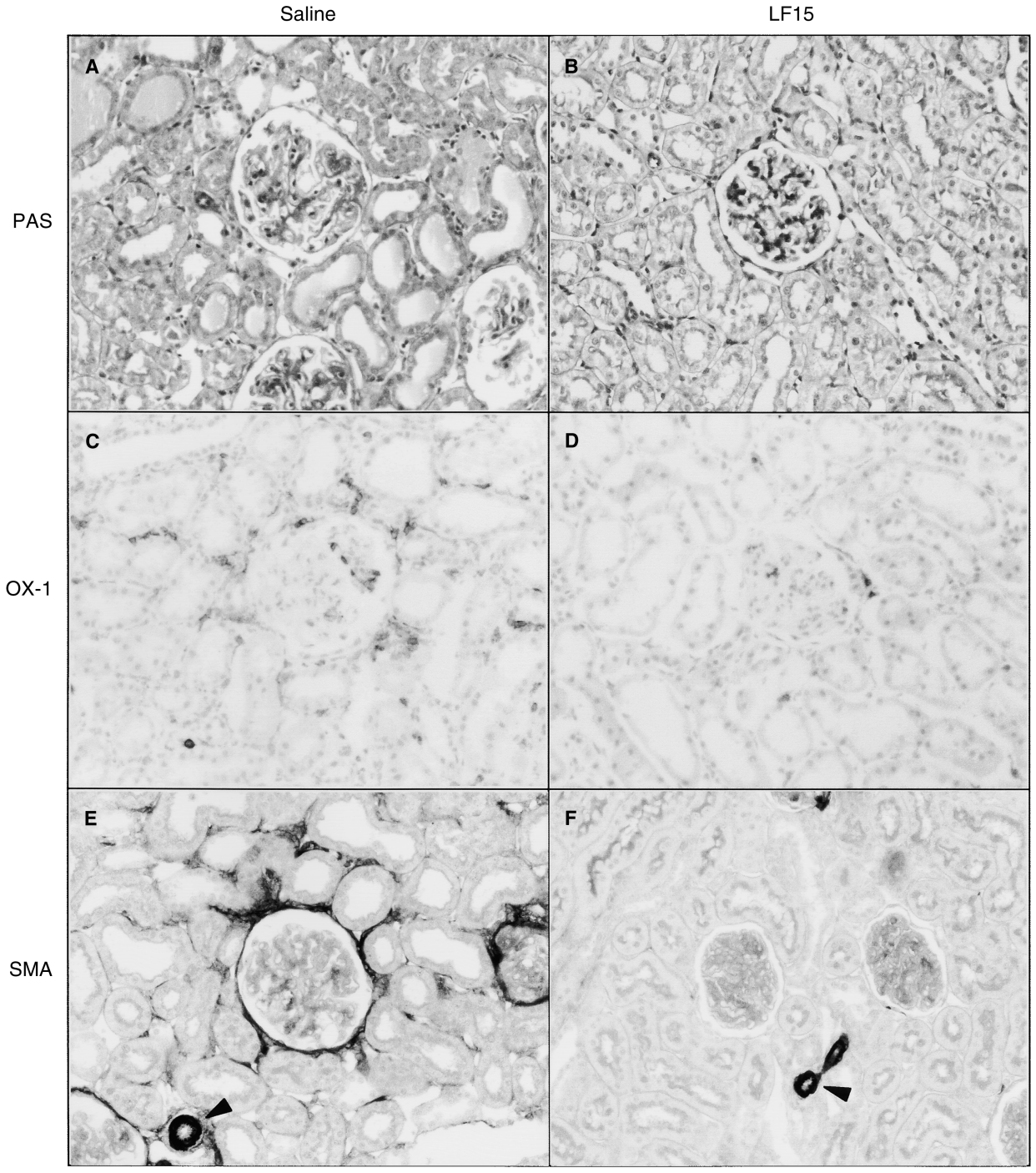


Fig. 4. LF15-0195 suppresses renal pathology during the induction of rat anti-GBM disease. Kidney sections stained with PAS and hematoxylin show the development of histological lesions in saline-treated rats at day 14 during the induction of anti-GBM disease (*A*), which were largely suppressed in rats that received LF15-0195 treatment (*B*). Immunoperoxidase staining of saline-treated rats shows infiltrating OX-1+ kidney leukocytes at day 14 (*C*), which were reduced by LF15-0195 treatment (*D*). Kidney myofibroblasts expressing α-smooth muscle actin in saline-treated rats at day 14 (*E*) were diminished by LF15-0195 treatment (*F*). Constitutive expression of α-smooth muscle actin in kidney vessels is indicated by arrowheads (E and F). Magnification A–F, ×100.

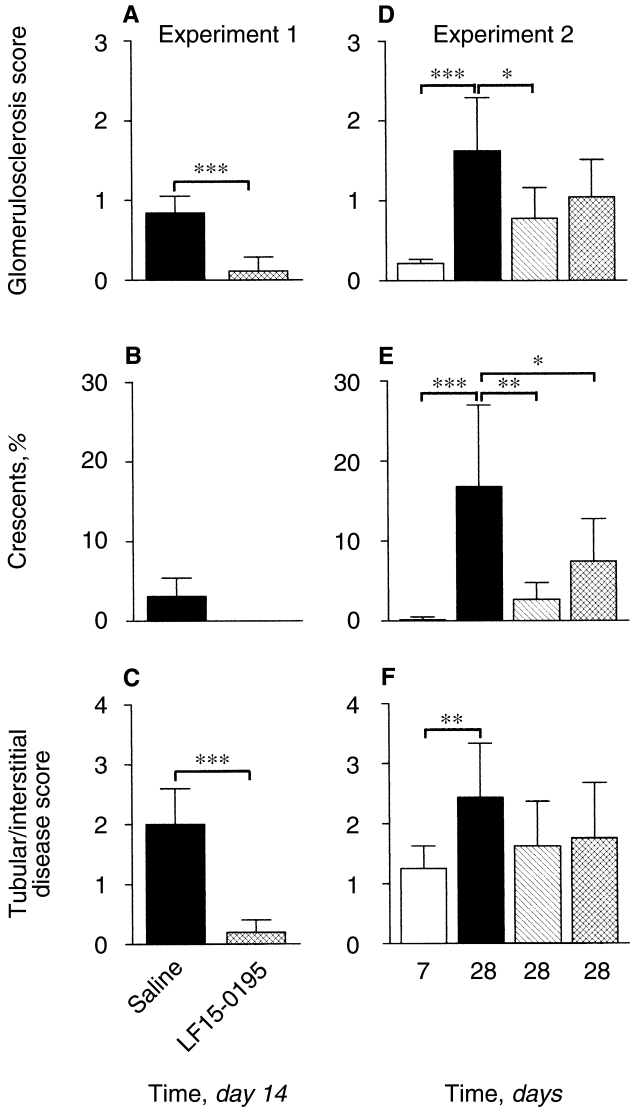


Fig. 5. LF15-0195 reduces histological lesions in anti-GBM disease. During the induction of anti-GBM disease, LF15-0195 treatment markedly inhibited the appearance of segmental glomerular lesions (*A*), glomerular crescents (*B*), and tubular/interstitial disease (*C*) at day 14 compared with saline-treated rats (experiment 1). Between days 7 and 28 of disease, there was an increase in glomerulosclerosis (*D*) and crescent formation (*E*), which was reduced by LF15-0195 intervention treatment (experiment 2). There was a semiquantitative increase in tubular/interstitial disease between days 7 and 28; however, LF15-0195 treatment did not significantly reduce this score (*F*). Data are mean ± SD, $N = 8$. Symbols are: (■) saline; (▨) pulse LF15-0195; (▩) continuous LF15-0195; (□) untreated; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

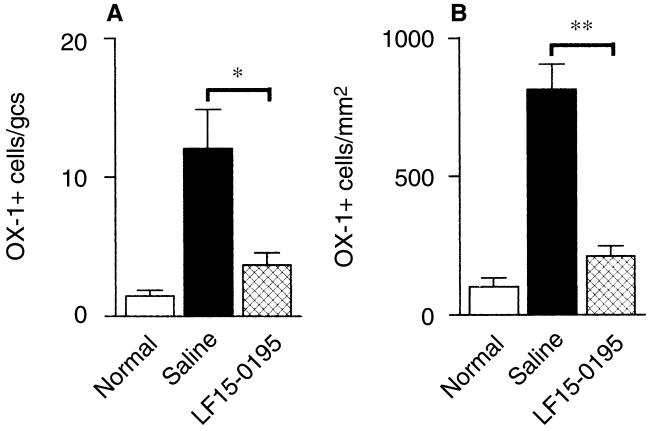


Fig. 6. LF15-0195 reduces kidney leukocyte accumulation in anti-GBM disease. Immunoperoxidase staining with OX-1 antibody was used to detect kidney leukocytes during anti-GBM disease. Increased numbers of leukocytes per glomerular cross-section (gcs) were detected in saline-treated rats at day 14 of disease compared with normal rats and were reduced to near-normal levels by daily LF15-0195 treatment commencing at the initiation of disease (*A*). LF15-0195 treatment similarly decreased the numbers of interstitial leukocytes at day 14 of disease (*B*). Data are mean ± SD; $N = 8$; $^*P < 0.01$; $^{**}P < 0.001$.

were not detected in normal rat urine. LF15-0195 markedly reduced the urine levels of IL-1β (↓61%, 0.79 ± 0.70 pg/24 h, $P < 0.05$) and TGF-β (↓95%, 0.17 ± 0.49 pg/24 h, $P < 0.005$) at day 14 compared with saline treatment.

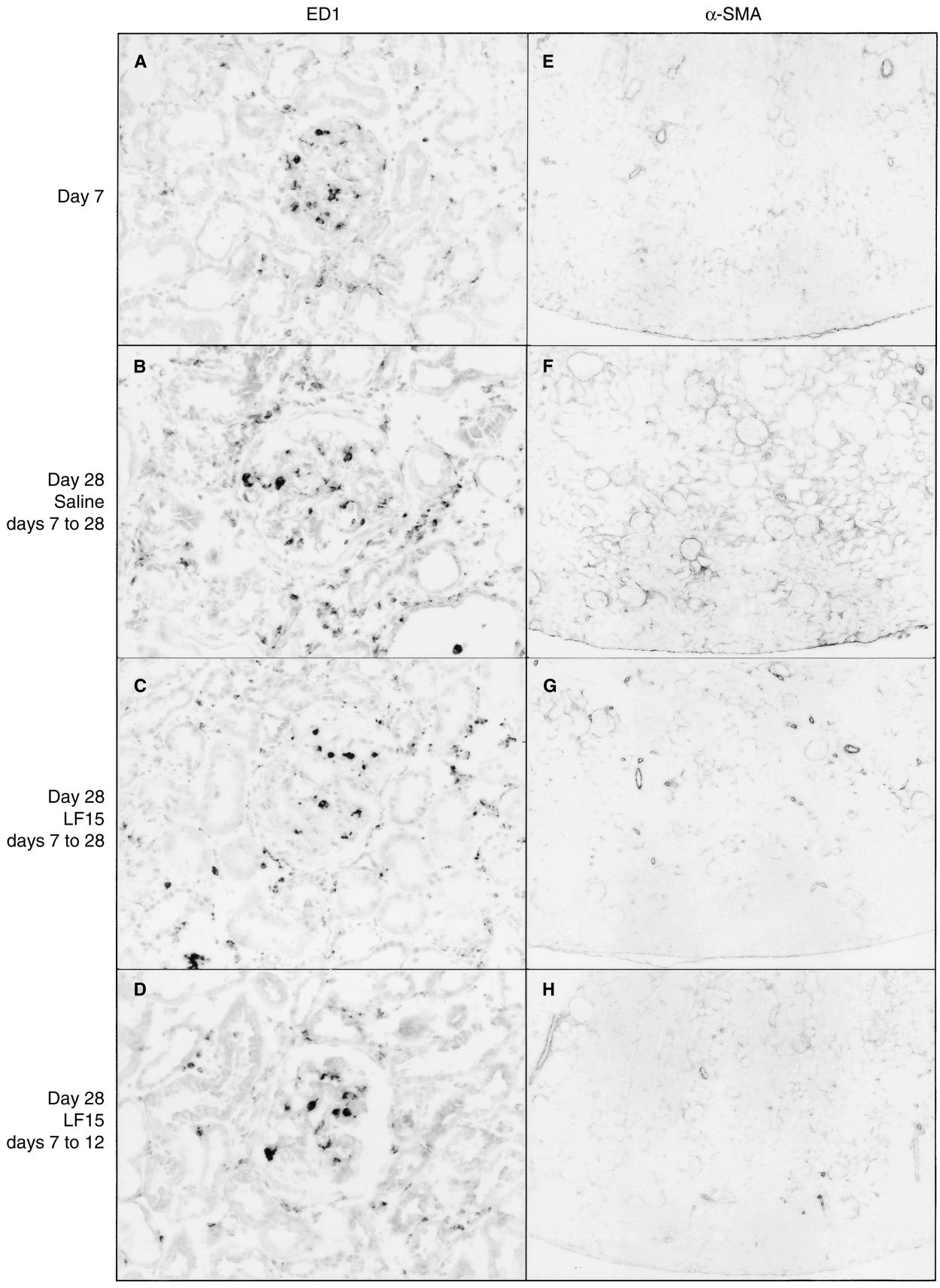
LF15-0195 inhibits the delayed-type hypersensitivity response

A single dose of LF15-1095 administered 2 hours before a skin DTH antigen challenge inhibited the 24-hour skin swelling in response to sheep IgG (2.42 ± 0.15 mm, ↓17%, $P < 0.05$) compared with saline treatment (2.91 ± 0.15 mm). Blood measurements at the time of skin DTH assessment indicated that WBC levels were not affected by LF15-0195 (16.3 ± 0.9 K/μL WBC) compared with saline treatment (17.6 ± 1.5 K/μL WBC).

LF15-0195 suppresses the progression of established anti-GBM disease

At day 7 of anti-GBM disease, prior to the commencement of intervention therapy, rats had developed elevated WBC (Fig. 2 E, I), proteinuria (Fig. 3C), reduced creatinine clearance (Fig. 3D), glomerular and interstitial

Fig. 7. LF15-0195 treatment of established anti-GBM disease inhibits the progression of renal pathology. Immunoperoxidase staining in anti-GBM kidneys showing (*A*) ED1+ macrophages and (*E*) myofibroblasts labeled with α-smooth muscle actin (SMA) at day 7, prior to intervention treatment. (*B*) Macrophages and (*F*) myofibroblasts at day 28 following saline-treatment. (*C*) Macrophages and (*G*) myofibroblasts at day 28 following continuous LF15-0195 treatment. (*D*) Macrophages and (*H*) myofibroblasts following pulse LF15-0195 treatment. Magnification A–D, ×100, and E–H, ×20).


ED1
α-SMA
A
E
Day 7
B
F
Day 28
Saline
days 7 to 28
C
G
Day 28
LF15
days 7 to 28
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H
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days 7 to 12

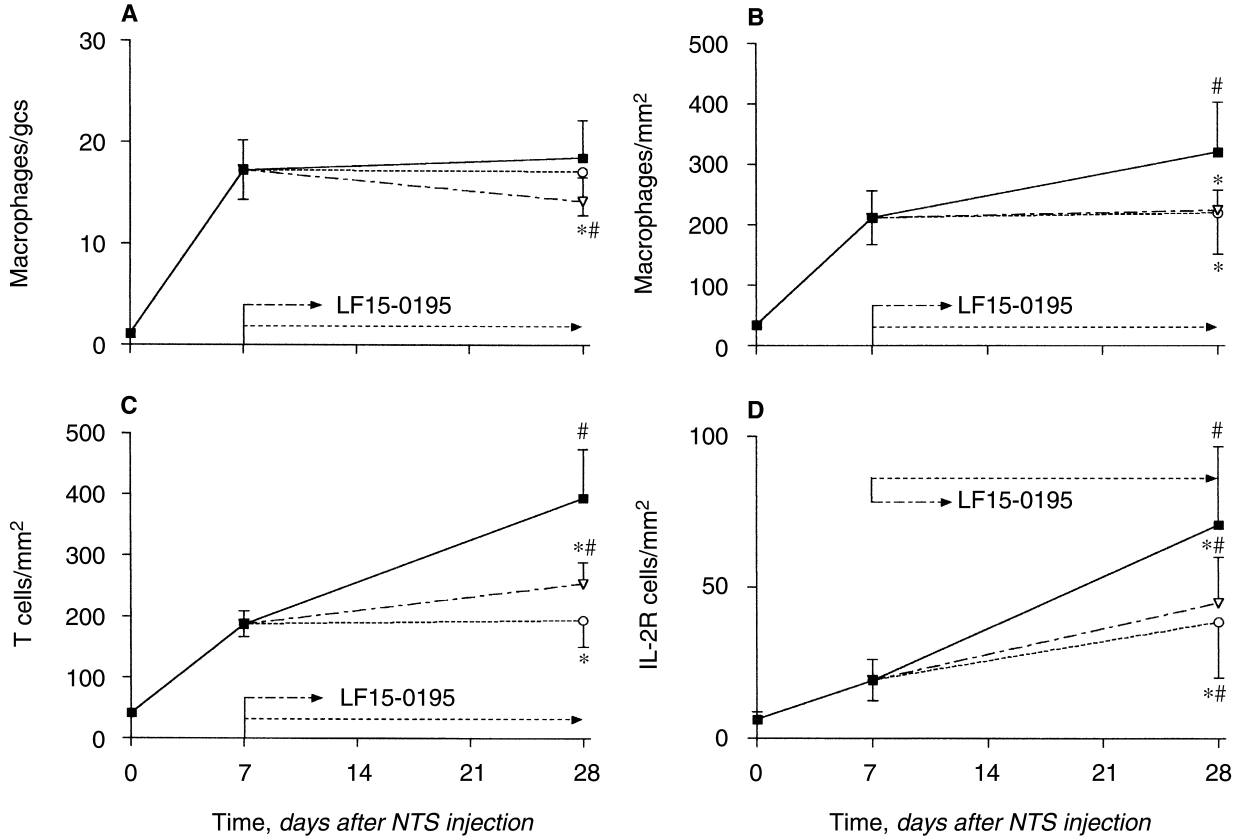

Fig. 8. LF15-0195 treatment of established anti-GBM disease suppresses the progressive accumulation of interstitial leukocytes. Immunoperoxidase staining was used to detect kidney macrophages (ED1+), T cells (R73+), and IL-2R cells (NDS-61+) during anti-GBM disease. Glomerular leukocytes consisted of almost entirely macrophages at day 7 of disease and did not increase by day 28. Glomerular macrophages were only marginally reduced by LF15-0195 treatment (*A*). In comparison, interstitial macrophages (*B*), T cells (*C*), and IL-2R cells (*D*) were all significantly increased between days 7 and 28 on disease. LF15-0195 treatment markedly suppressed the increase in these interstitial leukocytes (B–D). Data are mean ± SD; $N = 8$. Symbols are: (○) continuous LF15-0195; (▽) pulse LF15-0195; (■) saline; $*P < 0.05$ vs. day 28 saline-treated; $\#P < 0.05$ vs. day 7.

histological lesions (Fig. 5 D–F), a striking kidney macrophage infiltrate (Figs. 7A and 8 A, B), and a significant interstitial accumulation of myofibroblasts and collagen type III (Figs. 7B and 10).

Compared with saline-treated rats, those that received LF15-0195 continuously from days 7 to 28 of anti-GBM disease had reduced blood levels of neutrophils and monocytes, but not lymphocytes, at days 14, 21, and 28 (Fig. 2 E–H). Similarly, a five-day pulse treatment with LF15-0195 from days 7 to 12 lowered the numbers of blood neutrophils and monocytes at day 14 compared with saline treatment; however, all WBCs subsequently returned to the same level as saline-treated rats at day 21 (Fig. 2 I–H).

LF15-0195 intervention treatment partially inhibited the progressive increase in proteinuria and decrease in creatinine clearance that occurred between days 7 and 28 in saline-treated rats (Fig. 3 C, D). Both continuous and pulse LF15-0195 treatments were effective. Continuous LF15-0195 treatment prevented a progressive loss of creatinine clearance between days 7 and 28 and was significantly better than saline-treated rats at day 28. LF15-0195 pulse therapy was able to restore normal creatinine clearance at day 14, which thereafter declined and was not different to saline-treated rats at days 21 and 28.

In saline-treated rats at day 28, glomerular and interstitial lesions had progressed from moderate to severe. Crescents were observed in 16% of glomeruli, and infiltrating cells were significantly increased in the interstitium (Fig. 5 D–F). Both continuous and pulse LF15-0195 treatments inhibited the progression of histological lesions between days 7 and 28 (Fig. 5 D–F), resulting in reduced sclerosis and fewer glomerular crescents.

Between days 7 and 28 of anti-GBM disease, there was no increase in glomerular leukocytes, which consisted of mostly macrophages (>90%). Intervention treatment with LF15-0195 had little or no effect on glomerular macrophage accumulation (Figs. 7 A–D and 8A). In contrast, there was a significant increase in the number of

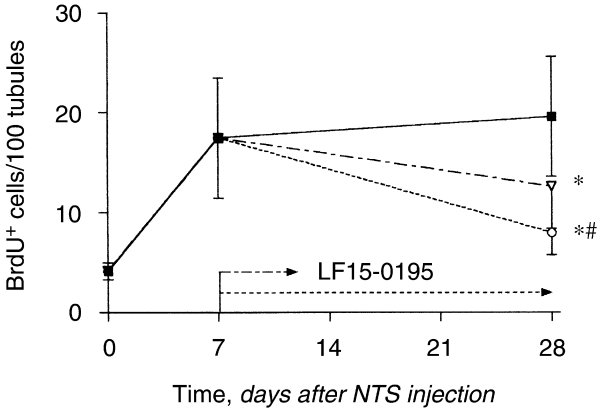

Fig. 9. LF15-0195 reduces tubular proliferation in established anti-GBM disease. Injured kidney tubules with proliferating epithelial cells were detected by immunostaining for bromodeoxyuridine (BrdU), which was injected into rats prior to killing. Continuous and pulse LF15-0195 treatment reduced the number of proliferating tubular cells compared with saline-treated animals. Data are mean ± SD, $N = 8$. Symbols are: (○) continuous LF15-0195; (▽) pulse LF15-0195; (■) saline; *$P < 0.01$ vs. day 28 saline treated; #$P < 0.05$ vs. day 7.

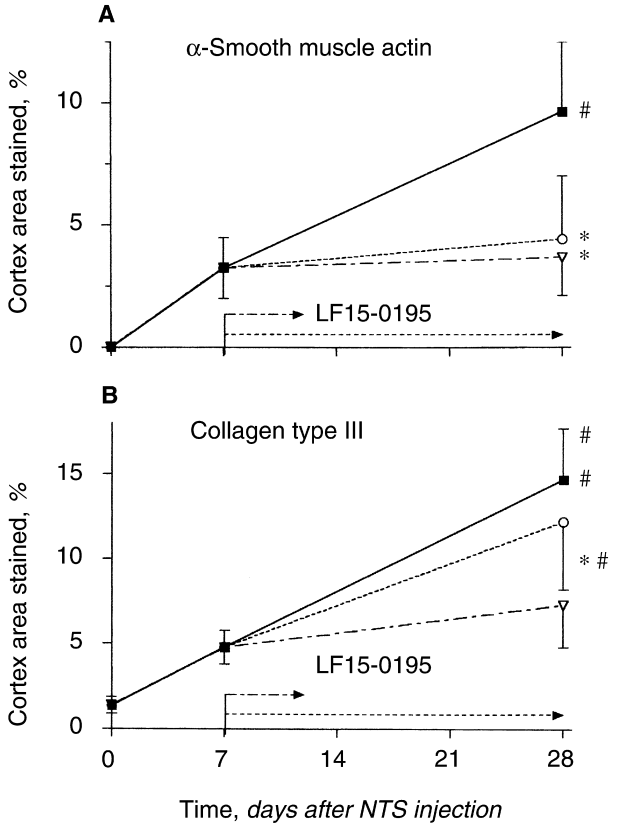

Fig. 10. LF15-0195 reduces the progression of interstitial fibrosis in established anti-GBM disease. Fibrosis in the interstitium of kidneys with anti-GBM disease was detected by immunoperoxidase staining for α-smooth muscle actin in myofibroblasts (*A*) and interstitial collagen type III (*B*). Pulse and continuous LF15-0195 intervention treatment prevented the progressive increase in interstitial α-smooth muscle actin expression between days 7 and 28. Pulse LF15-0195 treatment reduced collagen type III staining in the renal cortex at day 28 compared with saline treatment. Data are mean ± SD, $N = 8$. Symbols are: (○) continuous LF15-0195; (▽) pulse LF15-0195; (■) saline; *$P < 0.0005$ vs. day 28 saline-treated; #$P < 0.05$ vs. day 7.

macrophages, T cells and IL-2 receptor cells within the interstitium in saline-treated rats between days 7 and 28, which was markedly reduced by both pulse and continuous LF15-0195 treatment (Fig. 8 B–D).

Compared with normal rats, those with anti-GBM disease had a marked increase in tubular cell proliferation at day 7, which was maintained through to day 28 (Fig. 9). Both continuous and pulse LF15-0195 treatment reduced the number of proliferating BrdU+ tubular cells at day 28 compared with saline-treated rats. In addition, rats treated continuously with LF15-0195 from day 7 had fewer proliferating tubular cells at day 28 than at day 7 before treatment began.

Continuous and pulse LF15-0195 treatment prevented the increase in interstitial α-smooth muscle actin expression observed in saline-treated animals between days 7 and 28 (Fig. 10A). Compared with saline treatment, interstitial deposition of collagen type III was significantly reduced by pulse, but not continuous, LF15-0195 treatment at day 28 (Fig. 10B).

Following the initiation of LF15-0195 intervention treatment, there was a reduction of serum anti-sheep IgG observed at day 14 in both pulse and continuous treatment groups. However, the anti-sheep IgG level in the pulse treatment group returned to the same level as the saline-treated group at day 21, while continuous LF15-0195 treatment maintained serum anti-sheep IgG at the low level observed at day 14 (Fig. 11).

Urine TGF-β levels did not increase in saline-treated animals between days 7 (3.40 ± 1.53 pg/24 h) and 28 (4.49 ± 1.17 pg/24 h) of anti-GBM disease and were not reduced at day 28 by either continuous LF15-0195 treatment (3.94 ± 1.91 pg/24 h) or pulse LF15-0195 treatment (3.92 ± 1.32 pg/24 h). Urine IL-1β levels decreased between days 7 (3.50 ± 1.55 pg/24 h) and 28 (2.01 ± 1.39 pg/24 h, $P < 0.05$) and were not reduced at day 28 compared with saline treatment by either continuous LF15-0195 treatment (2.39 ± 1.84 pg/24 h) or pulse LF15-0195 treatment (2.37 ± 0.75 pg/24 h).

DISCUSSION

Our results demonstrate that LF15-0195 treatment can prevent the development of rat anti-GBM disease and can suppress the progression of this disease once it is already established. LF15-0195 averted the induction of anti-GBM disease by preventing the onset of proteinuria, loss of renal function, kidney leukocyte infiltration, the development of histological lesions, and renal fibrosis.

Continuous LF15-0195 treatment during disease induction reduced the serum levels of rat anti-sheep anti-

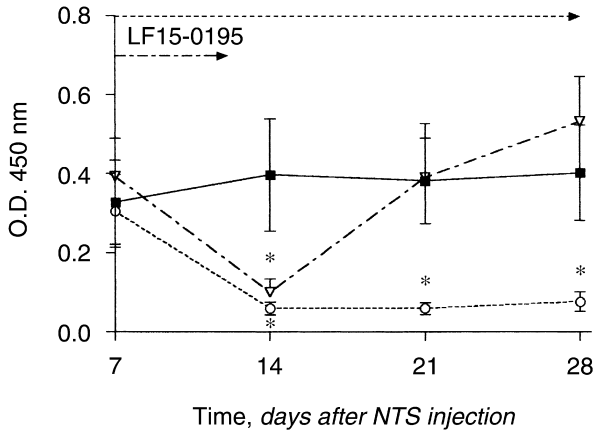

Fig. 11. LF15-0195 reduces serum antibodies in established anti-GBM disease. Serum levels of antibody against sheep IgG were determined by ELISA. The level of anti-sheep antibodies remained constant between days 7 and 28 in saline-treated rats. In comparison, rats that received continuous LF15-0195 treatment or pulse LF15-0195 treatment from day 7 had reduced serum antibodies at day 14. The reduction in serum antibodies was maintained in rats receiving continuous LF15-0195, while serum antibodies in pulse-treated rats returned to the same level as saline-treated at day 21. Data are mean ± SD; $N = 8$. Symbols are: (○) continuous LF15-0195; (▽) pulse LF15-0195; (■) saline; $^*P < 0.001$ vs. saline treated.

body at day 14, but did not significantly affect the glomerular deposition of sheep IgG, rat IgG, and C3. This suggests that the ability of LF15-0195 to prevent the induction of disease is not due to the prevention of the humoral response to glomerular deposited sheep IgG, but rather may reflect a suppression of a developing cell-mediated immune response. We noted that continuous LF15-0195 treatment decreased the levels of blood monocytes and neutrophils in rats at day 14; however, this reduction was not seen during the first 24 hours of treatment when induction of anti-GBM disease causes significant proteinuria, suggesting that LF15-0195 prevention of disease induction is not due to blood leukocyte depletion. Although it is not clear why LF15-0195 prevents the induction of rat anti-GBM disease, one possible explanation may be that LF15-0195 prevents the kidney recruitment and activation of leukocytes, which cause the initial glomerular injury resulting in proteinuria. This concept is supported by our finding that LF15-0195 treatment inhibits a skin DTH response without depleting WBC. In addition, a lack of urine IL-1β and TGF-β in LF15-0195–treated animals during induction of anti-GBM disease suggests that cytokines that can promote leukocyte-mediated tissue injury and fibrosis may not be present at high enough levels within the kidney to have pathological significance.

LF15-0195 treatment of established anti-GBM disease inhibited the progression of renal injury (increased proteinuria and decreased creatinine clearance) by suppressing the kidney interstitial accumulation of macrophages and T cells and subsequent tubular proliferation and renal fibrosis. This effect may be largely attributable to the suppression of interstitial leukocyte accumulation and activation, since macrophages and T cells can promote tubular injury and fibrotic responses, and their accumulation in the interstitium during kidney disease correlates with tubular responses to injury (that is, cell proliferation and apoptosis) and the development of renal fibrosis [18–21]. However, LF15-0195 also may have direct inhibitory effects on tubular and fibrotic responses within the kidney that could account for the reduction in tubular cell proliferation and α-smooth muscle actin expression following LF15-0195 treatment. The structurally related drug, deoxyspergualin, has been shown to inhibit the proliferation of mesangial cells [14], and therefore, LF15-0195 may suppress the proliferation of interstitial myofibroblasts or tubular epithelial cells through similar mechanisms. Studies have shown that the progression of renal injury during kidney disease is associated with the proliferation of tubular cells [22, 23] and the accumulation of interstitial myofibroblasts [24–27], indicating that LF15-0195–mediated inhibition of renal cell proliferation may be an important component in its suppression of the progression of kidney disease.

An interesting finding was that animals receiving pulse or continuous LF15-0195 treatment had similar reductions in some of the criteria used to assess the severity of renal disease (that is, proteinuria, leukocyte and myofibroblast accumulation, tubular cell proliferation, crescent formation). The similarities of outcomes between the pulse and continuous therapeutic strategies suggest that prolonged suppression of bone marrow associated with continuous LF15-0195 treatment may not be necessary for achieving an optimal reduction in kidney disease.

Of the immunosuppressants that have been used to treat the induction of anti-GBM disease, LF15-0195 appears to offer the greatest protection against renal injury. In comparison, treatment with deoxyspergualin can prevent a loss of renal function and suppress histological lesions, but only partially prevents proteinuria during the induction of anti-GBM disease [15]. CsA and FK506 can inhibit the development of accelerated anti-GBM disease when given prior to the Ig preimmunization. However, CsA treatment, after establishment of the antibody response, fails to alter the disease outcome [28, 29].

LF15-0195 is the first immunosuppressant that has been shown to be effective at suppressing the progression of established anti-GBM disease. Intervention treatment of accelerated anti-GBM disease with either azathioprine or methylprednisolone can partially reduce proteinuria and the humoral response, but is ineffective at reducing the development of histological lesions [30]. FK506, mycophenylate mofetil, and DSG are able to suppress partially the progression of established kidney disease in lupus mice [31–33], but it is not clear whether

these immunosuppressants would be effective in preventing the progression of the rapidly developing lesions associated with anti-GBM disease.

In conclusion, the immunosuppressant LF15-0195 is capable of suppressing the progression of established kidney disease and the loss of renal function during the development of accelerated rat anti-GBM nephritis. This finding suggests that LF15-0195 is a promising therapeutic reagent for the potential treatment of human glomerulonephritis.

ACKNOWLEDGMENT

This study was funded by Groupe Fournier, France.

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